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Metabolism of Dibenzo-p-Dioxin and Chlorinated Dibenzo-p-Dioxins by a Beijerinckia Species

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16. ABSTRACT
Whole cells of the parent strain of Beijerinckia, grown with succinate and biphenyl, oxidized dibenzo-p-dioxin and several chlorinated dioxins. The rate of oxidation of the chlorinated dibenzo-p-dioxons decreased with an increasing degree of chlorine substitution. A mutant strain (B8/36) of Beijerinckia oxidized dibenzo-p-dioxin to cis-1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin. The mutant organism also oxidized two monochlorinated dibenzo-p-dioxins to cis-dihydrodiols. No metabolites were detected from two dichlorinated dibenzo-p-dioxins. Growth of the parent strain of Beijerinckia was inhibited after 4 hr when 0.05% dibenzo-p-dioxin was present in the culture medium. Resting cell suspensions of the parent organism, previously grown with succinate and biphenyl, oxidized dibenzo-p-dioxin to a compound identified as 1,2-dihydroxydibenzo-p-dioxin. Further degradation of this metabolite was not detected, as the compound was found to be a potent mixed-type inhibitor of two ring-fission oxygenases present in this organism.

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Metabolism of Dibenzo-*p*-Dioxin and Chlorinated Dibenzo-*p*-Dioxins by a *Beijerinckia* Species

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Whole cells of the parent strain of *Beijerinckia*, grown with succinate and biphenyl, oxidized dibenzo-*p*-dioxin and several chlorinated dioxins. The rate of oxidation of the chlorinated dibenzo-*p*-dioxins decreased with an increasing degree of chlorine substitution. A mutant strain (B8/36) of *Beijerinckia* oxidized dibenzo-*p*-dioxin to *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin. The mutant organism also oxidized two monochlorinated dibenzo-*p*-dioxins to *cis*-dihydrodiols. No metabolites were detected from two dichlorinated dibenzo-*p*-dioxins. Growth of the parent strain of *Beijerinckia* on succinate was inhibited after 4 h when 0.05% dibenzo-*p*-dioxin was present in the culture medium. Resting cell suspensions of the parent organism, previously grown with succinate and biphenyl, oxidized dibenzo-*p*-dioxin to a compound identified as 1,2-dihydroxydibenzo-*p*-dioxin. Further degradation of this metabolite was not detected, as the compound was found to be a potent mixed-type inhibitor of two ring-fission oxygenases present in this organism.

Chlorinated dibenzo-*p*-dioxins have long been recognized as possible contaminants in the manufacture of various pesticides and herbicides derived from chlorinated phenols. The high toxicity of some of these contaminants, when considered in light of the extensive worldwide use of chlorophenols in both industry and agriculture, is of environmental concern.

The potential for the degradation of chlorinated dibenzo-*p*-dioxins in the environment has been investigated. Reductive dechlorination has been reported by several authors (6, 7). The reaction required ultraviolet (UV) light and the presence of a suitable organic hydrogen donor. Kearney et al. (15) have monitored the metabolism and persistence of ^{14}C-labeled 2,7-dichlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in experimental soil samples. 2,7-Dichlorodibenzo-*p*-dioxin was oxidized to $^{14}CO_2$ and to one or more polar metabolites detected in ethanol extracts of the soil samples. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was oxidized to a lesser extent, because over 50% of the original samples were recovered after 1 year. No metabolites were detected in soils treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Relatively little is known about the metabolism of these compounds by microorganisms. Matsumura and Benezet (19) reported the degradation of ^{14}C-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by soil organisms to unidentified polar metabolites. Klečka and Gibson (16) have recently reported the cooxidation of dibenzo-*p*-dioxin to *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin and 1,2-dihydroxydibenzo-*p*-dioxin by a *Pseudomonas* species (Fig. 1). Further oxidative metabolism was not detected.

This report describes the oxidation of dibenzo-*p*-dioxin and several chlorinated dibenzo-*p*-dioxins by a *Beijerinckia* species. Evidence is presented for the identity of metabolites before the enzymic fission of the aromatic nucleus. Further evidence is presented that describes the inhibition by 1,2-dihydroxydibenzo-*p*-dioxin of enzymes responsible for the oxidative fission of aromatic molecules.

MATERIALS AND METHODS

Organisms. The isolation of a *Beijerinckia* sp. has been reported (12). The organism was isolated from a polluted stream and has the ability to grow with biphenyl as a sole source of carbon. Treatment of the parent organism with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine led to the isolation of a mutant strain, *Beijerinckia* B8/36 that oxidizes several different aromatic hydrocarbons to *cis*-dihydrodiols (4, 11, 12, 14, 17).

Growth conditions. Organisms were maintained on agar slants of a mineral salts medium (22) containing 0.2% sodium succinate. Cells were transferred from a slant to 100 ml of mineral salts broth (pH 7.2) containing 0.2% succinate in a 500-ml Erlenmeyer flask. Cultures were incubated at 27°C on a rotary shaker at 150 rpm for 12 h. A 5% (vol/vol) inoculum of the culture described above was used for further experiments.

Biotransformation experiments were conducted in 2-liter Erlenmeyer flasks containing 500 ml of mineral salts medium as described above, with the exception that the phosphate buffering capacity was double

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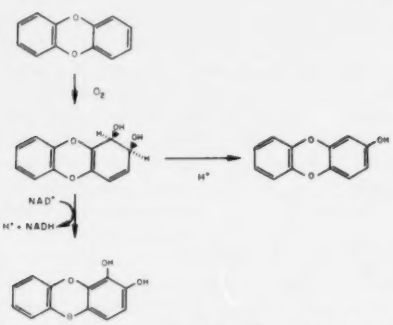

FIG. 1. *Pathway proposed for the oxidation of dibenzo-p-dioxin by Pseudomonas sp. N.C.I.B. 9816 (16).*

strength (pH 7.2). Succinate (0.2%) was included as the growth substrate. The various dibenzo-*p*-dioxins were added in 10.0 ml of acetone to give a final concentration of 0.05% in the culture medium. Acetone was not a growth substrate, nor did it inhibit the growth of the organism. Flasks were incubated as described above. Growth of the cultures was monitored turbidometrically at 600 nm. When samples of the cultures were filtered through glass wool, the dibenzo-*p*-dioxins did not interfere with the measurement of cell density.

Large-scale (10-liter) incubations of the parent strain of *Beijerinckia* were conducted essentially as described by Laborde and Gibson (17) in a New Brunswick model M14 Microferm fermentor. Biphenyl replaced dibenzothiophene as the inducing substrate. Cultures were filtered through glass wool to remove solid materials, and the cells were harvested with an air-driven Sharples continuous-flow centrifuge. The cell pellet was washed twice with 0.05 M KH_2PO_4 buffer, pH 7.2, and then resuspended in the same buffer (1 g of cells per 2 ml of buffer) for use in further studies.

Preparation of cell extracts. The parent strain of *Beijerinckia* was grown as described above in a large-scale culture containing 0.2% succinate and 0.05% biphenyl. Washed cells (5.0 g, wet weight) were suspended in 10.0 ml of 0.05 M KH_2PO_4 buffer, pH 7.2, containing 10% acetone. The suspension was subjected to sonic oscillation at 40 kHz with a Bronwill Biosonik III ultrasonic disintegrator for 1.5 min (three 30-s exposures). Cell debris was removed after centrifugation at 30,000 × *g* for 1 h at 5°C. The clear supernatant solution was used as a source of crude cell extract.

Detection of metabolites. Quantitative measurement of product accumulation in the culture medium was conducted at various time intervals. A 5.0-ml portion of the culture was extracted with 15.0 ml of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo at 30°C. The residue was dissolved in 0.5 ml of acetone and applied to a preparative thin-layer chromatography (TLC) plate, precoated with Silica Gel 60. The solvent system used for chromatography was chloroform-acetone (80:20). Compounds were located with the use of ultraviolet (UV) light. Regions of the plate containing suspected metabolites were removed and extracted with 5.0 ml of methanol. A sample (0.1 ml) of the methanol extract was diluted to 1.0 ml with the same solvent, and the UV spectrum was recorded. The reference cuvette contained methanol.

Alternatively, samples of duplicate culture extracts were examined by analytical TLC. After development of the chromatograms as described above, compounds were located with the use of UV light and also by spraying with a 2% (wt/vol) solution of 2,6-dichloroquinone-4-chloroimide in methanol (Gibbs reagent [9]). Subsequent exposure of the chromatogram to ammonia vapors often enhanced the color intensities of the different products.

Isolation of transformation products. When product accumulation in a culture had reached a maximum, the culture medium was filtered through glass wool to remove unused solid materials. The filtrate was extracted with 2 volumes of ethyl acetate, and the organic layer was dried over anhydrous Na_2SO_4. The solvent was removed in vacuo at 30°C. The residue was dissolved in 1.0 ml of acetone, applied to several preparative TLC plates, and chromatographed as described above. Products isolated from the chromatograms were further purified by recrystallization from hexane-acetone.

Analytical methods. UV and visible spectra were determined on a Beckman model 25 recording spectrophotometer. Infrared (IR) spectra were recorded on a Perkin-Elmer model 137 spectrophotometer and were referenced to the absorptions of polystyrene. Crystalline samples were mulled in Nujol and placed between NaCl disks. Low-resolution mass spectra were determined on a DuPont-Consolidated Electrodynamics Corp. model 21-491 spectrometer and were referenced to assigned perfluoroalkane peak fragments. Melting points were obtained with a Büchi melting point apparatus and are uncorrected.

Whole-cell protein determinations were conducted after digestion of a portion of the cell suspension in 0.1 N NaOH at 100°C for 1 h. Protein concentration was determined by the method of Lowry et al. (18), with crystalline bovine serum albumin as the standard.

Characterization of dihydrodiols. Evidence for the formation of a dihydrodiol was supported by the acid-catalyzed dehydration of the compound to phenols. Suspected metabolites were dissolved in 3.0 ml of methanol. The reaction was initiated with the addition of 10.0 μl of 5.0 N H_2SO_4. Formation of phenolic products in the reaction mixture was determined by analytical TLC and by monitoring changes in the UV absorption spectrum of a sample of the solution.

Preliminary evidence for a *cis*- configuration of dihydrodiols was obtained by the synthesis of an isopropylidine derivative (2). Dihydrodiols were suspended in 3.0 ml of 2,2-dimethoxypropane. The suspension was chilled in an ice bath before the addition of 0.1 mg of *p*-toluenesulfonic acid. The reaction was monitored by TLC. Formation of an isopropylidine derivative was inferred by detection of a nonpolar product in the reaction mixture.

Enzyme analyses. Determination of the rate of whole-cell oxidation of various substrates was conducted polarographically with a Clark-type oxygen electrode at 30°C, using 0.05 M KH_2PO_4 buffer, pH

7.0, which was saturated with air before use. The reaction mixture, in a total volume of 1.4 ml, contained buffer and a portion of a cell suspension. Reactions were initiated with the addition of 200 nmol of the substrate in 10.0 μl of *N,N*-dimethylformamide. All rates were corrected for endogenous respiration of the cell suspension.

The activity of catechol oxygenases in cell extracts was determined polarographically, using 0.05 M KH_2PO_4 buffer, pH 7.5. The reaction mixture, in a total volume of 1.4 ml, contained buffer and an amount of cell extract found to give a linear rate of O_2 consumption with respect to protein concentration. 2,3-Dihydroxybiphenyl oxygenase was assayed with the addition of 100 nmol of 2,3-dihydroxybiphenyl in 10.0 μl of *N,N*-dimethylformamide. Catechol oxygenase was assayed with the addition of 100 nmol of pyrocatechol (in 10.0 μl of *N,N*-dimethylformamide). All rates were corrected for endogenous respiration. Negligible autooxidation of the catechol substrates was observed under the assay conditions described.

Spectrophotometric assay conditions were established for the oxygenase which converts 2,3-dihydroxybiphenyl to a yellow ring-fission product. The compound has been identified as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (3) and was found to exhibit an absorption maximum at 435 nm (ϵ = 15,800 M^{-1} cm^{-1}, pH 7.5; D. T. Gibson, unpublished data). The reaction mixture, in a total volume of 1.0 ml, contained 0.05 M KH_2PO_4 buffer, pH 7.5, and an amount of cell extract found to give a linear rate of product formation with respect to protein concentration. Reactions were initiated with the addition of 100 nmol of 2,3-dihydroxybiphenyl. Catechol-2,3-dioxygenase (EC 1.13.1.2) was assayed in an analogous manner by the method of Nozaki et al. (20). The formation of α-hydroxymuconic semialdehyde from catechol was monitored at 375 nm.

Materials. Dibenzo-*p*-dioxin was synthesized by the procedure described by Gilman and Dietrich (13). 1-Chloro- and 2-chlorodibenzo-*p*-dioxin were synthesized by the method of Pohland and Yang (21). Chemical identity of the compounds was verified by IR and mass spectrometry. 2,3-Dichloro- and 2,7-dichlorodibenzo-*p*-dioxin were obtained from RFR Corp., Hope, R.I. 2,8-Dichloro- and 1,2,4-trichlorodibenzo-*p*-dioxin were obtained from the Food and Drug Administration, Washington, D.C. 2,3-Dihydroxybiphenyl was prepared as described by Gibson et al. (12). Samples of the biological metabolites *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin, 2-hydroxydibenzo-*p*-dioxin, and 1,2-dihydroxydibenzo-*p*-dioxin were obtained as previously described (16). Analytical TLC was performed, using Brinkman chromatogram sheets, type SIL G/UV. Preparative TLC was conducted, using glass plates (5.0 by 20.0 cm) coated with Silica Gel 60 F-254 (Brinkman Instruments, Inc., Westbury, N.Y.). Organic solvents were purified by vacuum distillation. Diethylaminoethyl-cellulose was obtained from Whatman, Inc., Clifton, N.J. All other materials were of the highest purity commercially available and were used without further purification.

RESULTS

Preliminary observations. *Beijerinckia* sp. was unable to utilize dibenzo-*p*-dioxin or any of the chlorinated dibenzo-*p*-dioxins as a source of carbon and energy for growth. The ability of whole cells of *Beijerinckia* sp., previously grown with succinate and biphenyl, to oxidize various aromatic compounds was determined polarographically (Table 1). Dibenzo-*p*-dioxin was oxidized at a lower rate than the monochlorinated dibenzo-*p*-dioxins. The rate of oxidation of the chlorinated dibenzo-*p*-dioxins decreased with an increasing degree of chlorine substitution.

Oxidation of dibenzo-*p*-dioxin (I) by *Beijerinckia* B8/36. When *Beijerinckia* B8/36 was grown in mineral salts medium containing 0.2% succinate and 0.05% dibenzo-*p*-dioxin (I), a neutral product (compound IA) was detected in the culture medium. Compound IA (R_f = 0.22) absorbed UV light and gave a brown color with Gibbs reagent. Growth of the culture and accumulation of compound IA with time are shown in Fig. 2. After 24 h, duplicate cultures were extracted to leave 5.8 mg of a solid residue. Purification of the residue led to the isolation of 3.0 mg of a crystalline product that melted at 135 to 136°C (compound IA). The metabolite had mass, IR and UV spectra identical to those of 1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin. This product was obtained from the cooxidation of dibenzo-*p*-dioxin by *Pseudomonas* sp. N.C.I.B. 9816 strain 11 (16). Further support for this structure was provided by the conversion of compound IA to 2-hydroxydibenzo-*p*-dioxin. Compound IA (2.0 mg) was suspended in 3.0 ml of methanol, and the reaction was initiated with the addition of 10.0 μl of 5.0 N H_2SO_4. After 30 min of incubation at 27°C, the solvent was removed, leaving 2.0 mg of a solid residue. TLC of the residue revealed that compound IA was converted to a phenolic product (compound IB; R_f = 0.5) that gave a purple color with Gibbs reagent. The latter compound was also detected in the cooxidation cultures after 20 h. Compound IB was identified as 2-hydroxydibenzo-*p*-dioxin,

TABLE 1. *Oxidation of substrates by washed whole cells of Beijerinckia sp.*

Substrate[a]	Sp act[b]	Relative rate (%)
Biphenyl	72.3	100.0
Dibenzo-*p*-dioxin	29.8	41.2
1-Chlorodibenzo-*p*-dioxin	52.5	72.6
2-Chlorodibenzo-*p*-dioxin	52.6	72.7
2,3-Dichlorodibenzo-*p*-dioxin	11.2	15.5
2,7-Dichlorodibenzo-*p*-dioxin	8.5	11.8
2,8-Dichlorodibenzo-*p*-dioxin	8.5	11.8
1,2,4-Trichlorodibenzo-*p*-dioxin	6.5	8.9

[a] All substrates added in *N,N*-dimethylformamide (200 nmol/10.0 μl).

[b] Oxygen uptake at 30°C (nmol of O_2 consumed per min per mg of protein).

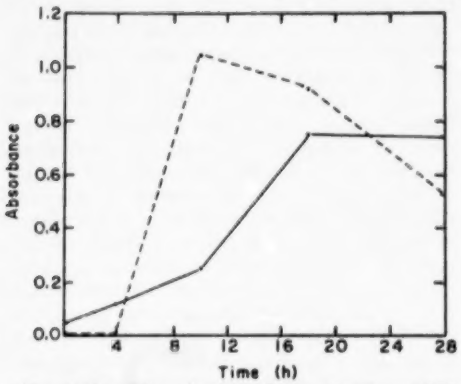

FIG. 2. *Growth and accumulation of compound IA by cultures of Beijerinckia B8/36. A 500-ml culture was grown as described in the text. Growth of the culture was monitored turbidometrically at 600 nm* (——). *Accumulation of compound IA was measured quantitatively as described in the text. Analysis of the UV spectrum of compound IA indicated an absorption maximum at 227 nm. Accumulation of compound IA with time is expressed as absorbance at 227 nm per milliliter of culture* (·····).

having a melting point, mass, and IR and UV spectra identical to those of the known standard. Preliminary evidence for a *cis*- configuration of compound IA was obtained with the synthesis of an isopropylidine derivative. Compound IA (1.0 mg) was suspended in 3.0 ml of 2,2-dimethoxypropane, and the reaction was initiated with the addition of 0.1 mg of *p*-toluenesulfonic acid. Analysis of the reaction mixture by TLC revealed the formation of a nonpolar product (compound IC; R_f = 0.64) that absorbed UV light and gave a yellow-brown color with Gibbs reagent. These results indicate that *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin is the first detectable metabolite of the oxidation of dibenzo-*p*-dioxin by *Beijerinckia* sp. This compound can subsequently undergo a nonenzymatic dehydration to yield 2-hydroxydibenzo-*p*-dioxin.

Oxidation of chlorinated dibenzo-*p*-dioxins by *Beijerinckia* B8/36. *Beijerinckia* B8/36 was grown in duplicate cultures as above with the exception that dibenzo-*p*-dioxin was replaced by 1-chloro- (II), 2-chloro- (III), 2,3-dichloro- (IV), and 2,7-dichloro- (V) dibenzo-*p*-dioxins. The accumulation of neutral products (compounds IIA and IIIA) was detected in cultures containing monochlorinated dibenzo-*p*-dioxins (Fig. 3). No products were detected in cultures grown in the presence of the two dichlorinated dibenzo-*p*-dioxins.

Cultures containing monochlorinated dibenzo-*p*-dioxins were extracted with ethyl acetate. Subsequent purification led to the isolation of 2.5 and 1.5 mg of compounds IIA and IIIA, respectively. The properties of the crystalline products are summarized in Table 2. The results indicate that the monochlorinated dibenzo-*p*-dioxins were oxidized to dihydrodiols. Further support for the formation of dihydrodiols was indicated by the acid-catalyzed dehydration of compounds IIA and IIIA to phenols (Fig. 4). Compound IIA was converted to a product

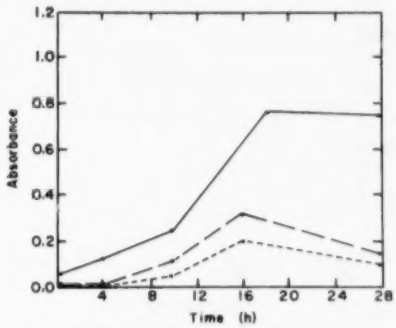

FIG. 3. *Growth and accumulation of compounds IIA and IIIA by cultures of Beijerinckia B8/36. Cultures (500 ml each) containing 1-chloro- (II) and 2-chlorodibenzo-p-dioxin (III) were grown as described in the text. Growth of the cultures was monitored turbidometrically at 600 nm* (——). *Accumulation of compounds IIA and IIIA was measured quantitatively as described in the text. Analysis of the UV spectrum of compound IIA indicated an absorption maximum at 235 nm. Compound IIIA exhibited an absorption maximum at 237 nm. The accumulation of compounds IIA and IIIA with time is expressed as absorbance at 235 nm* (– – –) *and at 237 nm* (·····) *per milliliter of culture, respectively.*

TABLE 2. *Physical properties of the metabolites formed from monochlorinated dibenzo-p-dioxins by Beijerinckia B8/36*

Property	Compound	
	IIA	IIIA
R_f (chloroform:acetone)	0.21	0.21
Gibbs reaction	Brown	Brown
Melting point	130 to 131°C	ND[a]
UV maxima (methanol)	235 nm	237 nm
	303 nm	309 nm
IR maxima (Nujol)	3.10 μm	3.07 μm
	5.95 μm	5.97 μm
	6.37 μm	6.37 μm
	9.45 μm	9.45 μm
Mass spectra (*m/e*)	252 (M^+)	252 (M^+)
	234 ($-H_2O$)	234 ($-H_2O$)
Number of Cl	1	1
Proposed structure	$C_{12}H_9O_4Cl$	$C_{12}H_9O_4Cl$

[a] ND, Not done.

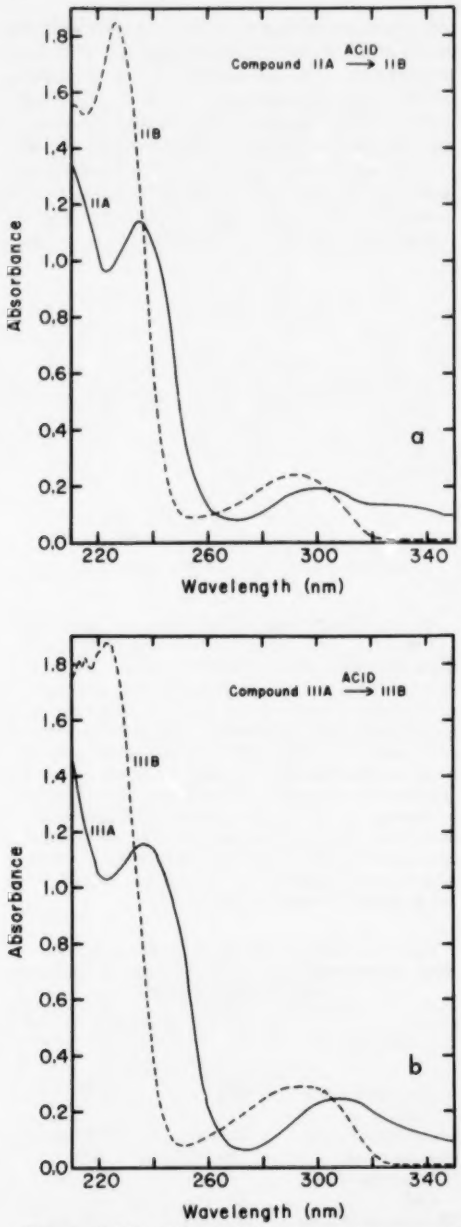

FIG. 4. *Acid-catalyzed dehydration of compounds IIA and IIIA. UV absorption spectra of compounds IIA and IIIA was monitored before (——) and after (- - - - -) acidification with H_2SO_4 as described in the text. Each compound was isolated and purified by preparative TLC and recrystallization from hexane-acetone. Ccmpounds IIA and IIIA (1.0 mg) were dissolved in 3.0 ml of methanol, and the UV spectrum was recorded. Dehydration of compounds IIA and IIIA was initiated with the addition of 10.0 μl of 5.0 N H_2SO_4, and the solutions were incubated at 27°C for 30 min. Reactions were monitored by TLC as described in the text. Upon completion of the acid-catalyzed dehydration, the UV spectrum was again recorded.*

(compound IIB; R_f = 0.5) that absorbed UV light and gave a purple color with Gibbs reagent. Compound IIIA was also converted to a phenolic product (compound IIIB; R_f = 0.51) that absorbed UV light and gave a dark blue color with Gibbs reagent. Additional products were not detected in the reaction mixtures. Preliminary evidence for a *cis*- stereochemistry of the hydroxyl groups of the dihydrodiols (compounds IIA and IIIA) was obtained with the syntheses of isopropylidine derivatives. Compounds IIA and IIIA were converted to nonpolar products, compounds IIC (R_f = 0.62) and IIIC (R_f = 0.62), respectively, when incubated in the presence of 2,2-dimethoxypropane as described above. Both of the latter products absorbed UV light and gave tan colors with Gibbs reagent. The isopropylidine derivatives of compounds IIA and IIIA were found to be highly unstable and decomposed to products resembling compounds IIB and IIIB. These results indicate that *cis*-dihydrodiols are also the initial metabolites from the oxidation of monochlorinated dibenzo-*p*-dioxins by *Beijerinckia* sp.

Oxidation of dibenzo-*p*-dioxin (I) by *Beijerinckia* sp., wild type. When the parent strain of *Beijerinckia* was grown in mineral salts medium containing 0.2% succinate and 0.05% dibenzo-*p*-dioxin (I), no products were detected by TLC or spectrophotometric analysis of the culture medium. In addition, the growth of the organism was inhibited after 4 h (Fig. 5). To determine whether further oxidation was possible, a large-scale culture of *Beijerinckia* sp. was grown as described with 0.2% succinate and 0.05% biphenyl. The washed cell pellet (5.0 g, wet weight) was suspended in 500 ml of 0.05 M KH_2PO_4 buffer, pH 7.2, containing 0.05% dibenzo-*p*-dioxin. The resting cell suspension was incubated on a rotary shaker at 27°C. After 12 h, the suspension was filtered through glass wool to remove unused dibenzo-*p*-dioxin. The filtrate was adjusted to pH 4.0 with concentrated H_2SO_4 and extracted with two volumes of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed, leaving 21.0 mg of an oily residue. Analysis of the residue by TLC revealed the formation of a neutral product (compound ID; R_f = 0.3) that absorbed UV light and gave an intense brown color with Gibbs reagent. Purification of the residue led to

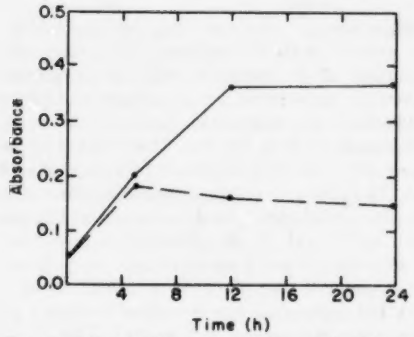

FIG. 5. *Effect of dibenzo-p-dioxin on the growth of Beijerinckia sp., wild type. Cultures (500 ml each) of the parent strain of Beijerinckia were grown in mineral salts broth with 0.2% succinate as the carbon source. Experimental cultures contained 0.05% dibenzo-p-dioxin. Growth of control (——) and experimental (-----) cultures was monitored turbidometrically at 600 nm.*

the isolation of 6.0 mg of a crystalline product that melted at 172 to 173°C. Compound ID was identified as 1,2-dihydroxydibenzo-*p*-dioxin, having mass, IR, and UV spectra identical to those of the biological product previously characterized (16).

Oxidation of 1,2-dihydroxydibenzo-*p*-dioxin by cell extracts. Polarographic experiments with cell extracts were conducted to test the ability of 1,2-dihydroxydibenzo-*p*-dioxin to serve as a substrate for enzymes responsible for the oxidative fission of the aromatic nucleus (Fig. 6). An initial investigation revealed that the cell extracts were active on the physiological substrates, 2,3-dihydroxybiphenyl (64.1 nmol of O_2 consumed per min per mg of protein) and catechol (212.2 nmol of O_2 consumed per min per mg of protein). Cell extracts exhibited a brief initial rate of oxidation of 1,2-dihydroxydibenzo-*p*-dioxin (98.6 nmol of O_2 consumed per min per mg of protein) and then rapidly fell to the nonenzymatic rate. In addition, incubation of cell extracts (0.81 mg of protein) with as low as 10 nmol of 1,2-dihydroxydibenzo-*p*-dioxin led to complete inhibition or inactivation of the catechol and 2,3-dihydroxybiphenyl oxygenases, because additions of 100 nmol of either substrate did not result in further enzymatic activity.

To determine whether both enzymes catalyzing the oxidative fission of the aromatic nucleus were capable of oxidizing 1,2-dihydroxydibenzo-*p*-dioxin, the 2,3-dihydroxybiphenyl oxygenase was separated from the catechol oxygenase by ion-exchange chromatography (Fig. 7). A crude cell extract (0.23 g of protein) was loaded onto a column of diethylaminoethyl-cellulose (1.4 by 8.0 cm) which had been previously equilibrated with 0.05 M KH_2PO_4 buffer, pH 7.5, containing 10% acetone (phosphate-acetone buffer). The column was washed with 50 ml of phosphate-acetone buffer, and 10-ml fractions were collected. The two activities were eluted separately, using a stepwise KCl gradient in phosphate-acetone buffer. 2,3-Dihydroxybiphenyl oxygenase was eluted with 0.2 M KCl in phosphate-acetone buffer (fractions 18 through 21) and exhibited activity with both 2,3-dihydroxybiphenyl and catechol. The catechol oxygenase was eluted with 0.4 M KCl in phosphate-acetone buffer (fractions 23 through 28). Fractions from each peak with the highest specific activity were pooled and utilized in further studies.

To determine whether the activity present in fractions 18 through 21 was due to a single protein species, the effect on activity of incubation at 55°C with time was investigated. The activity in the fraction for both 2,3-dihydroxybiphenyl and catechol was found to decrease at the same rate with time. Thus, it was assumed that the activities were due to the action of a single enzyme, which was tentatively identified as the 2,3-dihydroxybiphenyl oxygenase.

Polarographic experiments were performed to

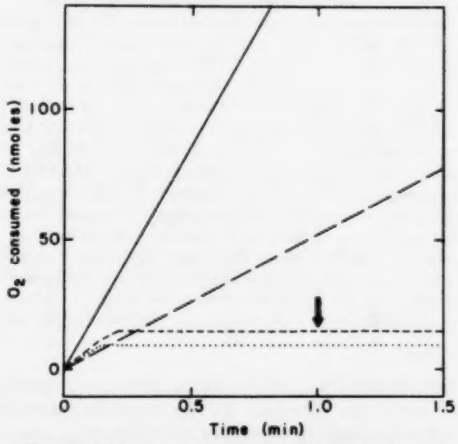

FIG. 6. *Polarographic assay of ring-fission oxygenases in cell extracts of Beijerinckia sp. The reaction vessel contained, in a total volume of 1.4 ml, 0.05 M KH_2PO_4 buffer, pH 7.5, and cell extract (0.81 mg of protein). The reaction was initiated with the addition of 100 nmol of catechol (——), 100 nmol of 2,3-dihydroxybiphenyl (– – –), 100 nmol of 1,2-dihydroxydibenzo-p-dioxin (-----), and 10 nmol of 1,2-dihydroxydibenzo-p-dioxin (·····), all in 10.0 μl of N,N-dimethylformamide. At the time indicated by the arrow (↓), 100 nmoles of catechol or 2,3-dihydroxybiphenyl was added. All rates were corrected for endogenous respiration. No autooxidation of the catechol substrates was observed under the assay conditions described.*

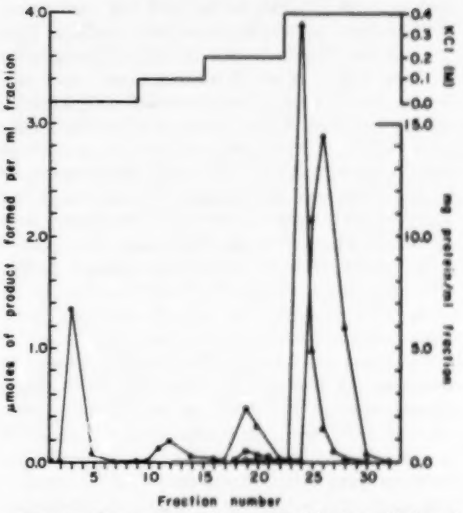

FIG. 7. *Separation of the 2,3-dihydroxybiphenyl and catechol oxygenases by ion-exchange (diethylaminoethyl) chromatography. A cell extract (20 ml) was prepared from Beijerinckia sp. as described in the text. The extract (0.23 g of protein) was loaded onto a column of diethylaminoethyl-cellulose (1.4 by 8.0 cm) which had been previously equilibrated with 0.05 M KH_2PO_4 buffer, pH 7.5, containing 10% acetone (phosphate-acetone buffer). The column was washed with 50 ml of phosphate-acetone buffer, and 10-ml fractions were collected. Protein was eluted from the column with a stepwise gradient of 60 ml of 0.1 M KCl in phosphate-acetone buffer, 70 ml of 0.2 M KCl in phosphate-acetone buffer, and 120 ml of 0.4 M KCl in phosphate-acetone buffer. Samples (10 to 100 μl) of each fraction were analyzed spectrophotometrically for oxygenase activity with catechol (▲) and 2,3-dihydroxybiphenyl (■) as described in the text. Enzyme activity is expressed as micromoles of product formed per minute per milliliter of fraction. Protein concentration (●) in the fractions was determined by the method of Lowry et al. (18).*

test the ability of 1,2-dihydroxydibenzo-*p*-dioxin to serve as a substrate for the partially purified 2,3-dihydroxybiphenyl and catechol oxygenases. The results of the analyses are summarized in Table 3. The 2,3-dihydroxybiphenyl oxygenase (0.48 mg of protein) was capable of oxidizing both 1,2-dihydroxydibenzo-*p*-dioxin and 2,3-dihydroxybiphenyl, whereas the catechol oxygenase (0.54 mg of protein) was unable to oxidize 1,2-dihydroxydibenzo-*p*-dioxin.

Spectrophotometric assays were utilized for kinetic analyses to determine the type of inhibition exhibited by 1,2-dihydroxydibenzo-*p*-dioxin with respect to the physiological substrates of the partially purified 2,3-dihydroxybiphenyl and catechol oxygenases. All inhibition studies were conducted after preincubation of the enzyme with the inhibitor for 1 min before initiation of the reaction with the appropriate substrate. Activity of the 2,3-dihydroxybiphenyl oxygenase was monitored, using a protein concentration of 0.12 mg/ml. The Michaelis constant (K_m) for 2,3-dihydroxybiphenyl was 8.3 μM. The effect of various concentrations of 2,3-dihydroxybiphenyl was determined in the presence of 1.0 μM 1,2-dihydroxydibenzo-*p*-dioxin. Results of the analyses were used to prepare a Lineweaver-Burke plot (Fig. 8a). Activity of the catechol oxygenase was monitored, using a protein concentration of 0.2 mg/ml. The K_m for catechol was found to be 7.1 μM. The effect of various concentrations of catechol was determined in the presence of 0.4 μM 1,2-dihydroxydibenzo-*p*-dioxin. A Lineweaver-Burke plot was prepared from the results (Fig. 8b). These investigations indicate that 1,2-dihydroxydibenzo-*p*-dioxin is a potent mixed-type inhibitor of both the 2,3-dihydroxybiphenyl and catechol oxygenases present in this organism.

DISCUSSION

Beijerinckia sp. was unable to utilize dibenzo-*p*-dioxin or any of the chlorinated dibenzo-*p*-dioxins as a source of carbon and energy for growth. However, cooxidation was observed under certain conditions. Whole cells of the parent strain of *Beijerinckia*, previously grown on succinate with biphenyl as an inducing substrate, oxidized dibenzo-*p*-dioxin and several mono-, di-, and trichlorinated dibenzo-*p*-dioxins. The fact that monochlorinated dibenzo-*p*-dioxins were oxidized faster than the parent compound may reflect substrate or product inhibition of the enzymes systems involved. Further studies are needed to clarify this point. *Beijerinckia* B8/36 was able to cooxidize dibenzo-*p*-dioxin and two monochlorinated dibenzo-*p*-dioxins when grown on sodium succinate. However, no

TABLE 3. *Polarographic analysis of the activities of the partially purified 2,3-dihydroxybiphenyl and catechol oxygenases*

Oxygenase	Substrate[a]	Sp act[b]
2,3-Dihydroxybiphenyl	2,3-Dihydroxybiphenyl	35.6
	1,2-Dihydroxydibenzo-*p*-dioxin	64.4[c]
Catechol	Pyrocatechol	206.9
	1,2-Dihydroxydibenzo-*p*-dioxin	0.0

[a] All substrates added in *N,N*-dimethylformamide (100 nmol/10.0 μl).

[b] Specific activity (nanomoles of O_2 consumed per minute per milligram of protein).

[c] Initial rate—fell rapidly to nonenzymatic level.

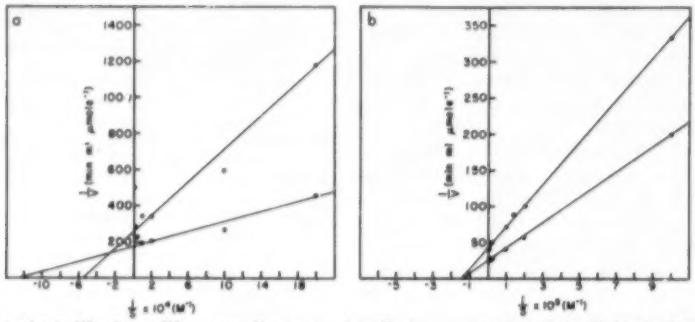

FIG. 8. *Effect of 1,2-dihydroxydibenzo-p-dioxin on ring-fission oxygenases from Beijerinckia sp. (a) Effect of 1,2-dihydroxydibenzo-p-dioxin on 2,3-dihydroxybiphenyl oxygenase. A protein concentration of 0.12 mg/ml was used in the analysis. The enzyme was preincubated in the presence of 1.0 μM 1,2-dihydroxydibenzo-p-dioxin for 1 min before initiation of the reaction with substrate. Activity of the 2,3-dihydroxybiphenyl oxygenase was determined at various concentrations of 2,3-dihydroxybiphenyl in the presence (○) and absence (●) of the inhibitor. (b) Effect of 1,2-dihydroxydibenzo-p-dioxin on catechol oxygenase. A protein concentration of 0.2 mg/ml was used in the analysis. The enzyme was preincubated in the presence of 0.4 μM 1,2-dihydroxydibenzo-p-dioxin for 1 min before initiation of the reaction with substrate. Activity of the catechol oxygenase was determined at various concentrations of catechol in the presence (○) and absence (●) of the inhibitor.*

products were detected in cultures grown in the presence of two dichlorinated dibenzo-*p*-dioxins. These results indicate that dibenzo-*p*-dioxin and monochlorinated dibenzo-*p*-dioxins induce the synthesis of enzymes responsible for oxidation of the aromatic nucleus in the *Beijerinckia* sp. Furthermore, it appears that the dichlorinated dibenzo-*p*-dioxins fail to function as inducing substrates. Thus, the degree of chlorine substitution on the aromatic ring may have an effect on the ability of a compound to induce the synthesis of degradative enzymes in this organism. In contrast, previous studies have demonstrated that dibenzo-*p*-dioxin failed to induce the synthesis of enzymes necessary for aromatic hydrocarbon degradation in *Pseudomonas* sp. N.C.I.B. 9816 (16).

Bacteria initiate the oxidation of many aromatic hydrocarbons by the enzymatic incorporation of one molecule of oxygen into the aromatic nucleus to form dihydrodiols with a *cis*-stereochemistry (10). The oxidation of dibenzo-*p*-dioxin and two chlorinated dibenzo-*p*-dioxins by a *Beijerinckia* sp. is consistent with this observation. The present study has demonstrated that *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin is the first detectable metabolite of the oxidation of dibenzo-*p*-dioxin by a *Beijerinckia* sp. An identical metabolite was previously isolated from a *Pseudomonas* sp. (16). It should be noted that the *cis*- stereochemistry of the hydroxyl groups was inferred from evidence for the formation of an isopropylidine derivative with 2,2-dimethoxypropane (2) and also by the known ability of *Beijerinckia* B8/36 to form *cis*-dihydrodiols with other substrates (4, 11, 12, 14, 17). The acid-catalyzed dehydration of the *cis*-dihydrodiols of dibenzo-*p*-dioxin and the monochlorinated dibenzo-*p*-dioxins occurs rapidly and leads to the formation of phenols. It is of interest that the acid-catalyzed dehydration of the dibenzo-*p*-dioxin dihydrodiols appears to result in the formation of single phenolic products. This phenomenon may be due to resonance stabilization or conformation of the dehydration intermediates and hence may favor the formation of single phenolic products (1, 14).

Subsequent oxidation of *cis*-dihydrodiols leads to the formation of catechols which are substrates for ring-fission dioxygenases and ultimately leads to complete biodegradation (5, 8). The conversion of *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin to 1,2-dihydroxydibenzo-*p*-dioxin is consistent with the first part of this statement. However, it is noteworthy that the further degradation of 1,2-dihydroxydibenzo-*p*-dioxin does not appear to be favored due to the inhibition and possibly the inactivation of the ring-fission dioxygenases by this metabolite. A similar phenomenon was observed during the oxidation of dibenzo-*p*-dioxin by *Pseudomonas* sp. N.C.I.B. 9816 (16). The present investigation has revealed that 1,2-dihydroxydibenzo-*p*-dioxin is a potent mixed-type inhibitor of both the 2,3-dihydroxybiphenyl and catechol oxygenases present in the *Beijerinckia* sp. The nature of this inhibitory phenomenon is under current investigation.

It is of interest that growth of the parent strain of *Beijerinckia* on succinate was inhibited when

dibenzo-*p*-dioxin (0.05%) was included in the culture medium. As *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin was not inhibitory for the growth of the mutant strain (B8/36) of *Beijerinckia*, the toxic effects may be due to the oxidation of dibenzo-*p*-dioxin to 1,2-dihydroxydibenzo-*p*-dioxin. This metabolite was found to accumulate when resting cell suspensions of the parent strain of *Beijerinckia* were incubated in the presence of dibenzo-*p*-dioxin. Furthermore, growth inhibition of the parent strain was observed after 4 h. This period corresponds approximately to the amount of time required by the organism for induction of enzymes necessary for aromatic hydrocarbon oxidation, as indicated by the accumulation of *cis*-1,2-dihydroxy-1,2-dihydrodibenzo-*p*-dioxin after 4 h by *Beijerinckia* B8/36.

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